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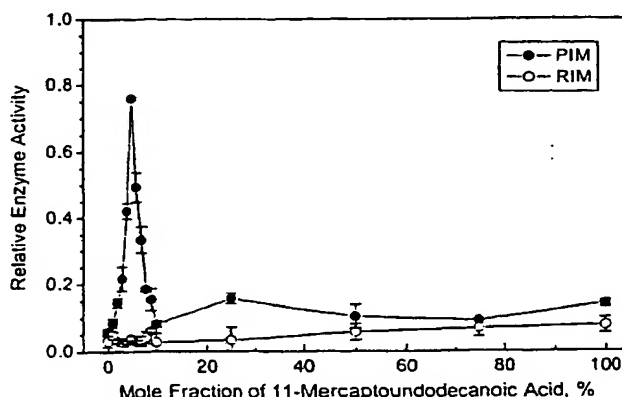
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(54) Title: A METHOD FOR IMMOBILIZING MOLECULES WITH PHYSIOLOGICAL ACTIVITY



(57) Abstract: The present invention relates to an efficient immobilization method that can maximally preserve biological activity of a physiologically active molecule by masking the active site (region) of the physiologically active molecule. The method for immobilizing a physiologically active molecule of the present invention comprises the steps of (a) reacting the physiologically active molecule with a masking compound that selectively binds to the active site so as to mask the active site; (b) forming a supporting material by introducing on the substrate material a linker that will bind to the masked physiologically active molecule prepared in step (a); (c) controlling the rate of the immobilization reaction in which the masked physiologically active molecule prepared in step (a) binds to the linker on the supporting material formed in step (b); and (d) immobilizing the masked physiologically active molecule prepared in step (a) on the supporting material by reacting with the linker on the supporting material formed in step (b). The method can further comprise step (d) of removing the masking compound from the masked physiologically active molecule. The immobilization reaction rate is optimized in the present invention by controlling mole fraction of the reaction group on the supporting material, concentration (or number of moles) of the physiologically active molecule, pH of the reaction solution, reaction time, reaction temperature, and type of a coupling reagent during the immobilization reaction.

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A METHOD FOR IMMOBILIZING MOLECULES WITH PHYSIOLOGICAL ACTIVITY

TECHNICAL FIELD

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The present invention relates to a method for immobilizing physiologically (or biologically) active molecules on the surface of a supporting material and the physiologically active molecules immobilized thereby. More particularly, it relates to an efficient immobilization method that can maximally preserve the physiological activity of the immobilized molecules by masking active site of the physiologically active molecules during the immobilization process, and the physiologically active molecules immobilized using the method.

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BACKGROUND ART

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Recently, the effort for identifying and/or probing activities of physiologically active molecules such as nucleic acids, proteins, enzymes, antibodies, antigens, and the like by combining various biotechnologies and semiconductor manufacturing technologies is proliferating worldwide. Immobilization of desired physiologically active molecules on a small silicon or glass chip within specific areas of micro size and biochemical assay thereafter allow to obtain useful information efficiently. Efficient immobilization methods are required in developing biochips, Lab-on-a-chip, etc. for diagnosis, drug screening, and research, and also in enhancing efficiency of various biochemical assay processes that include separation, purification, and recycling of physiologically active molecules.

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Commonly used current methods utilize unspecific chemical bonding for

immobilization of the physiologically active molecules. In such methods, linker molecules each having a reaction group are introduced on a substrate material and chemical bonds are formed between multiple reaction groups of the linker molecules and multiple reaction groups of the physiologically active molecule. In the immobilization reaction, the physiologically active molecules are immobilized on the supporting material through a variety of bonding and binding such as covalent bonding, ionic bonding, coordination bonding, hydrogen bonding, packing, etc. using various reaction groups such as amine, carboxyl, alcohol, aldehyde, thiol, etc, that exist on the surface of the physiologically active molecules. Also, these physiologically active molecules have a single or multiple active sites for forming complexes with particular compounds such as substrate, coenzyme, antigen, antibody, etc.

For example, in one of the most utilized immobilization methods, the linker molecule having a reaction group is introduced onto a supporting material by physical or chemical adsorption, and the reaction groups of the linker molecules are activated to induce immobilization reaction with the physiologically active molecules. For example, carboxyl can be activated to react with primary amine using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxysuccinimide (NHS) in the presence of EDC, or SOCl_2 . Therefore, physiologically active molecules can be immobilized by reacting the activated carboxyl groups of the linker molecules with primary amines on the surface of physiologically active molecule (or protein). (*Anal. Biochem.*, vol. 185, pp. 131-135, 1990; *Anal. Chem.*, vol. 66, pp. 1369-1377, 1994; *Biosens. Bioelectron.*, vol. 11, pp. 757-768, 1996; *Biosens. Bioelectron.*, vol. 12, pp. 977-989, 1997; *Science*, vol. 289, pp. 1760-1763, 2000)

However, when a physiologically active molecule is immobilized by unspecific

chemical bonding as in the above example, there exist following problems. Firstly, since a plurality of reaction groups exist on the surface of the physiologically active molecule as well as on the supporting material, a plurality of immobilization bonding can be formed between the physiologically active molecule and the supporting material. Since a plurality of immobilization bonding can be unspecifically formed at various regions of the physiologically active molecule, there occur structural change and destruction of the physiologically active molecule upon immobilization, thereby causing undesirable reduction or destruction of the activity of the physiologically active molecule. Secondly, due to the unspecific nature of the immobilization reaction, in other words, as a plurality of reaction groups in the physiologically active molecule do not react selectively, the immobilization bonding can be formed directly at or near the active site. These chemical bonding at or near the active site can directly damage the active site, thereby reducing or destroying the activity of the physiologically active molecule after immobilization.

The immobilization methods using such unspecific chemical bonding give rise to damage in the active site and the molecular structure change in the physiologically active molecule, thereby reducing the activity per immobilized molecule and thus resulting in decrease of the overall activity per unit area of immobilization.

Therefore, it is required to develop a method for immobilizing physiologically active molecules in which direct chemical bonding at or near the active site and formation of multiple immobilization bonding can be avoided, so that the activity of the physiologically active molecule can be preserved efficiently after immobilization, thereby increasing the activity preservation ratio.

DISCLOSURE OF INVENTION

The present invention provides a method for immobilizing physiologically active molecules that does not give rise to steric hindrance or structural change in active site by means of masking the active site of the physiologically active molecules during immobilization reaction.

The present invention therefore provides a method that can improve the activity preservation ratio of the immobilized physiologically active molecules, thereby enhancing the overall activity per unit area of immobilization.

The present invention also provides a method for immobilizing physiologically active molecules that is useful in developing biochips or DNA chips.

Furthermore, the present invention provides immobilized physiologically active molecules that represents high activity preservation ratio.

The efficient immobilization method of the present invention that can maximally preserve the activity of a physiologically active molecule comprises the steps of: (a) reacting the physiologically active molecule with a masking compound that selectively binds to the active site so as to mask the active site; (b) forming a supporting material by introducing on a substrate material a linker that will bind to the masked physiologically active molecule prepared in step (a); (c) controlling the rate of the immobilization reaction in which the masked physiologically active molecule prepared in step (a) binds to the linker on the supporting material formed in step (b); and (d) immobilizing the masked physiologically active molecule prepared in step (a) on the supporting material by reacting with the linker on the supporting material formed in step (b).

Step (a) of the present invention where the active site of the physiologically active

molecule is masked, is a step where the masking compound that binds selectively to the active site of the physiologically active molecule reacts with the physiologically active molecule or with its active site, thereby forming a complex, a masked physiologically active molecule. This masking step can be performed before or simultaneously with step (d), where the physiologically active molecule is immobilized by reacting with the reaction group of the linker.

Examples of the physiologically active molecule include protein, enzyme, antigen, antibody, etc. The masking compound that can be used for masking the active site of the physiologically active molecule, can be selected from the group consisting of substrate, inhibitor, cofactor, their chemically modified compound, their homolog and their derivative for masking enzyme; antibody and its modification for masking antigen; and antigen and its modification for masking antibody. For example, an enzyme whose substrate is DNA or RNA can be masked by DNA, RNA, their derivative, or their homolog. Antibody can be masked by antigen or its derivative or homolog, and similarly antigen by antibody or its derivative or homolog; e.g. anti-DNA antibody can be masked by DNA as used in one of the examples described in the present invention.

The masking compound binds to one or more active sites or cofactor sites of the physiologically active molecule to form a complex. The complex can be formed through covalent bonding, ionic bonding, coordination bonding, hydrogen bonding, dipole-dipole interaction, packing, or the combination of two or more of such bonding or binding. The reaction time of complex formation can vary from several seconds to a day. The reaction pH is not specifically limited, as far as the activity of the physiologically active molecule is not destructed and complex formation for masking the active site can thus take place efficiently at the given pH. The masking ratio, i.e., ratio of the masked amount to total

amount of the physiologically active molecule, can be selected preferably within the range 5 ~ 100%.

Formation of immobilization bonding at or near the active site can be prevented by masking the active site of the physiologically active molecules with a masking compound (for example substrate or inhibitor for enzyme) that selectively binds to the active site, as described in step (a).

The physiologically active molecule whose active site is masked can be immobilized on the supporting material, that is, a substrate material where a plurality of the reaction groups for immobilization are introduced. The substrate material herein means a material on which a plurality of the reaction groups can be introduced within the size range comparable to the size of the physiologically active molecule.

The reaction groups are typically introduced on the surface of the substrate material by forming a thin film of the linker comprising a reaction group. The linker that forms a thin film on the substrate material has a reaction group to bind to the substrate material by covalent bonding, ionic bonding, coordination bonding, hydrogen bonding, packing, or the combination of two or more of such bonding or binding. Examples of the reaction group of the linker that reacts with the substrate material include thiol, sulfide, disulfide, silane such as alkoxysilane and halogen silane, carboxyl, amine, alcohol, epoxy, aldehyde, alkylhalide, alkyl, alkene, alkyne, aryl, or the combination of two or more of such reaction groups.

The substrate material that can be used for the present invention includes metal such as Au, Ag, Pt, Cu, etc., non-metal such as silicon wafer, glass, silica, and fused silica, semiconductor, oxide of such elements, organic or inorganic macromolecule, dendrimer, polymer of solid or liquid phase, and their mixture. The substrate material can be fabricated to various shape and morphology such as planar, spherical, linear, or porous form, a

microfabricated gel pad, a nano-particle, etc. The substrate material can include any material with various shape and morphology, as far as its size is larger than or equal to several nm and it is thus possible to introduce a plurality of the reaction groups for immobilization on its surface. Because the size of the physiologically active molecule is on the nm range that is an order of magnitude larger than the atomic distance on the Å range, any substrate material of nm size or larger can be used in the present invention as far as a plurality of the reaction groups can be introduced on its surface.

Examples of the reaction groups of the linker that react with the physiologically active molecules include carboxyl, amine, alcohol, epoxy, aldehyde, thiol, sulfide, disulfide, alkyl halide, alkyl, alkene, alkyne, aryl, or the combination of two or more of such groups. These reaction groups can react to connect the masked physiologically active molecules to the supporting material.

The bonding between the linker and the physiologically active molecule can also be covalent bonding, ionic bonding, coordination bonding, hydrogen bonding, or their combination as in the bonding between the linker and the substrate material. The immobilization bonding can be amide bonding, imine bonding, sulfide bonding, disulfide bonding, ester bonding, ether bonding, amine bonding, or the combination of two or more of such bonding. For example, amine of the physiologically active molecule and carboxyl of the linker or vice versa can react to form amide bonding, amine of the physiologically active molecule and aldehyde of the linker or vice versa to form imine bonding, and thiol of the physiologically active molecule and thiol of the supporting material to form disulfide bonding.

Even though the active site of the physiologically active molecule is masked with the masking compound, structural change in the physiologically active molecule could occur to

damage or destruct the activity of the immobilized physiologically active molecule if too many immobilization bonding can be formed. In this case, reduction in the activity due to formation of the multiple immobilization bonding can be prevented by down-kinetic-regulation, that is, by reducing the rate of the immobilization reaction and thus reducing the probability of the immobilization reaction. However, when down-kinetic-regulation is excessive, the overall activity per unit area of immobilization can decrease due to reduction in the probability of immobilizing the physiologically active molecule. Therefore, it is essential to optimize the rate of the immobilization reaction by controlling the kinetic variables such that the probability of forming multiple immobilization bonding for each physiologically active molecule is reduced, while keeping the probability of immobilizing the physiologically active molecule as high as possible. The reaction rate is optimized in the present invention by controlling mole fraction of the reaction group on the substrate material, concentration of the physiologically active molecule, pH of the reaction solution, reaction time, reaction temperature, and type of the coupling reagent.

For example, the mole fraction of the reaction group is controlled in the present invention by introducing two different thiol molecules having two different terminal groups onto the surface of the supporting material. One of the thiol molecules has the reaction group for immobilization in its terminal and a longer alkyl chain, while the other has a non-reactive group, different from the reaction group for immobilization, and a shorter alkyl chain. The latter thiol molecule is used to mask the supporting material against the immobilization reaction. The former thiol molecule having the reaction group is selected from the group consisting of mercaptocarboxylic acid such as 11-mercaptoundecanoic acid, mercaptoaminoalkane, mercaptoaldehyde, dimercaptoaldehyde, dimercaptoalkane, and sulfide and disulfide having a reaction group such as carboxyl, thiol, alcohol, aldehyde,

amine, etc. The latter thiol molecule having the non-reactive group can be selected from the group consisting of mercaptoalcohol such as 6-mercapto-1-hexanol, mercaptoalkane such as 1-heptanethiol, and sulfide or disulfide having a non-reactive group. It is preferable that the thiol molecule having the reaction group is mercaptocarboxylic acid or mercaptoaminoalkane and the thiol molecule having the non-reactive group is mercaptoalcohol or mercaptoalkane; that the former molecule is mercaptoaldehyde and the latter molecule is mercaptoalcohol or mercaptoalkane; and that the former molecule is dimercaptoalkane and the latter molecule is mercaptoalcohol or mercaptoalkane.

The mole fraction of the linker molecule having the reaction group for immobilization is preferably about 0.05 ~ 50% and more preferably about 0.05 ~ 30%. When the mole fraction of the linker molecule having the reaction group is too high, for example in excess of 50%, formation of multiple immobilization bonding can damage the activity of the immobilized physiologically active molecules. When it is too low, for example less than 0.05%, the probability of immobilization decreases. Therefore, the overall activity per unit area of immobilization decreases in such too high or too low mole fraction ranges.

The reaction group introduced on the supporting material, for example carboxyl, can be activated by a coupling reagent, for example 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxysuccinimide (NHS) in the presence of EDC, SOCl_2 , etc. The activated reaction group then reacts with the masked physiologically active molecule.

The concentration of the physiologically active molecule for optimizing the immobilization reaction is in the range 0.1 $\mu\text{g/ml}$ ~ 1 mg/ml . The pH of the immobilization reaction is in the range 4 ~ 10, and the immobilization reaction time is in the range of several seconds to 24 hours.

The method for immobilizing the physiologically active molecule provided in the present invention can further include step (e) where the masking compound that is bound to the active site of the immobilized physiologically active molecule is removed. By removing the masking compound and exposing the active site, change in the active site due to binding of the masking compound can be recovered, and thus it is possible to obtain a highly preserved activity for the immobilized physiologically active molecule. The masking compound can be removed by heating, hydrolysis, dilution, dialysis, pH change, etc.

In the present invention, the physiologically active molecules whose active site are masked, are used and the rate of the immobilization reaction is optimized in order to minimize the number of immobilization bonding per physiologically active molecule, while keeping the probability of immobilizing the physiologically active molecule as high as possible. This in turn prevents or minimizes damage in the activity of the immobilized physiologically active molecule and therefore increases the activity preservation ratio, thereby maximizing the overall activity per unit area of immobilization.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1a shows change in the activity of immobilized *Taq* DNA polymerase using the protected (or masked) immobilization method (PIM) according to the present invention and the random immobilization method (RIM) according to the prior art. The agarose gel fluorescence photographs in this figure show the activity change in each case as a function of the mole fraction of 11-mercaptoundecanoic acid in the mixed thiol solution used to introduce the carboxyl group as the reaction group for immobilization.

Figure 1b is a graph showing the relative activity of immobilized *Taq* DNA

polymerase using the PIM of the present invention and the RIM of the prior art, as a function of the mole fraction of 11-mercaptoundodecanoic acid in the mixed thiol solution used to introduce the carboxyl group as the reaction group for immobilization.

Figure 2a is an agarose gel fluorescence photograph of the polymerase chain reaction (PCR) products and it shows the activity of the immobilized *Taq* DNA polymerase as a function of the active site masking ratio for forming the DNA-*Taq* DNA polymerase complex.

Figure 2b is a graph showing the activity change of the immobilized *Taq* DNA polymerase as a function of the active site masking ratio when a partially double stranded DNA and *Taq* DNA polymerase form a 1:1 complex.

Figure 3a is an agarose gel fluorescence photograph of the PCR products showing the activity of the immobilized *Taq* DNA polymerase as a function of pH of the immobilization reaction.

Figure 3b is a graph showing the activity change of the immobilized *Taq* DNA polymerase as a function of pH of the immobilization reaction.

Figure 4a is an agarose gel fluorescence photograph of the PCR products showing the activity of the immobilized *Taq* DNA polymerase depending on immobilization reaction time.

Figure 4b is a graph showing the activity change of the immobilized *Taq* DNA polymerase as a function of immobilization reaction time.

Figure 5a is an agarose gel fluorescence photograph of the PCR products comparing the activity of the immobilized *Taq* DNA polymerase and that of the *Taq* DNA polymerase in solution as a function of number of the PCR cycle.

Figure 5b is a graph comparing the activity of the immobilized *Taq* DNA polymerase

and that of the solution phase *Taq* DNA polymerase as a function of number of the PCR cycles.

Figure 6a is an agarose gel fluorescence photograph of the PCR products and it shows the activity of the immobilized *Taq* DNA polymerase as a function of total amount of the *Taq* DNA polymerase used in the immobilization reaction.

Figure 6b is a graph showing the activity change of the immobilized *Taq* DNA polymerase as a function of total amount of the *Taq* DNA polymerase used in the immobilization reaction.

Figure 7 is a graph showing the activity of the immobilized anti-DNA antibody as a function of mole fraction of 11-mercaptoundecanoic acid in the mixed thiol solution used to introduce the carboxyl group as the reaction group for immobilization.

Figure 8 is a graph showing the activity of the anti-DNA antibody as a function of number of moles of the antigenic double stranded DNA.

BEST MODE FOR CARRYING OUT THE INVENTION

The present invention is explained in detail using the following examples, though the examples are only illustrative but not limiting the scope of the present invention.

Example 1: Immobilization of *Taq* DNA polymerase

a) Masking of the active site of *Taq* DNA polymerase

Taq DNA polymerase was purchased from Perkin Elmer (AmpliTMTaq GoldTM). This DNA polymerase is an chemically modified enzyme with molecular weight of 94 kDa consisting of 832 amino acids that can be activated by heating, for example by placing for

10 minutes at 95°C.

A 65 base single stranded DNA (ss-DNA) and the KS primer shown below was mixed in an aqueous buffer solution at 1:1 molar ratio, and the resulting solution was incubated for 10 minutes at 94°C and was then cooled down slowly below 35°C in a period of 1~2 hours. During this process, the 65 base ss-DNA and the KS primer were annealed to generate a partially double stranded DNA. A desired amount of the *Taq* DNA polymerase was then added to this solution and the resulting mixture was incubated in a dry bath at 72°C for 10 minutes. The mixture was then moved to a dry bath at 50°C and incubated for 20 minutes to prepare the reaction solution of the masked *Taq* DNA polymerase. In the masked *Taq* DNA polymerase, *Taq* DNA polymerase is bound to the 3' terminal region of the short KS primer of the partially double stranded DNA, where the DNA structure changes from a double strand to a single strand (See S. H. Eom, J. Wang, T. A. Steitz, *Nature*, vol.382, pp.278-281, 1996). This leads to masking of the active site of the *Taq* DNA polymerase. The 65 base ss-DNA and the KS primer used in this process were synthesized using a DNA synthesizer. The optimal pH for masking the active site was found to be 8.3, at which the activity of the *Taq* DNA polymerase was known to be the highest.

KS primer

5' CGAGGTCGACGGTATCG 3'

3' CCAGCTGCCATAGCTATTTTCTTTTCTTTCTTAAGTTCTTTTCTTTTCCTAGG

TGATCAAGATCT 5'

b) Formation of the monolayer of thiol molecules on the surface of the Au substrate and

introduction of the reaction group

The Au substrate used was a glass plate of 3.0 mm×5.0 mm size on which Au was vacuum-deposited to about 1000 Å thickness. In order to ensure the cleanness of the surface of the Au thin film, it was washed with Piranha solution for 10~15 minutes at 60
5 ~70°C right before using, and it was rinsed with deionized water and subsequently with absolute ethanol.

In order to introduce the immobilization reaction groups on the Au surface, a monolayer of thiol molecules was formed on the Au surface by using the Au-S bond formation reaction, that is, by using the thiolate formation reaction between the linker
10 having a thiol group and Au, to prepare a supporting material (C. B. Bain, E. B. Troughton, Y.-T. Tao, J. Evall, G. M. Whitesides, and R. G. Nuzzo, *J. Am. Chem. Soc.*, vol.111, pp.321-335, 1989). In this step, the mixed solution of two kinds of thiol molecules each having an immobilization reaction group and a non-reactive group was used. The mole fraction of the thiol molecule having the immobilization reaction group was controlled by changing its
15 mole fraction in the range 0~100%, in order to control the mole fraction of the immobilization reaction group on the supporting material. In order to introduce an carboxyl immobilization reaction group, 11-mercaptoundecanoic acid with a relatively longer alkyl chain was used. As a thiol molecule having a non-reactive group, 6-mercapto-1-hexanol was used. The Au thin film was placed in 100 µl of a 2 mM mixed thiol solution in
20 ethanol for 2 hours at room temperature to introduce the carboxyl reaction group, and it was then washed with absolute ethanol.

Since the immobilization reaction groups are spatially separated and protrudes out from the surface of the substrate material in the present example, motion of the immobilized physiologically active molecule becomes relatively un-restricted and also

molecular interactions between the immobilized physiologically active molecule and the supporting material can be minimized, leading to increased activity preservation ratio.

c) Activation of the carboxyl reaction group on the monolayer of the thiol molecules

5 The Au thin film where the carboxyl reaction groups were introduced was placed in 120 μ l of an ethanol solution containing 10 mM of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 5 mM of N-hydroxysuccinimide (NHS) for 2 hours at room temperature to activate the carboxyl group. The carboxyl group reacts with NHS in the presence of EDC to form NHS-ester (Z. Grabarek and J. Gergely, *Anal. Biochem.*, vol.185, pp.131-135, 1990), thereby being activated.

d) Immobilization reaction of *Taq* DNA polymerase

15 After activating the carboxyl group on the monolayer, the Au substrate was moved to the solution of the masked *Taq* DNA polymerase for immobilization reaction. In this step, the activated carboxyl (NHS-ester) on the monolayer reacted with the primary amine (-NH₂) of the protein to form amide bond (-CO-NH-) (Z. Grabarek and J. Gergely, *Anal. Biochem.*, vol.185, pp.131-135, 1990; V.M. Mirsky, M. Riepl, and O. S. Wolfbeis, *Biosens. Bioelectron.*, vol.12, pp977-989, 1997). As a result, the *Taq* DNA polymerase was immobilized on the substrate material. The immobilization reaction was carried out at
20 different conditions by varying concentration of the DNA polymerase, pH, reaction time, reaction temperature, etc.

Example 2: Immobilization of anti-DNA antibody

a) Masking of the active sites of anti-DNA antibody

The anti-DNA antibody is a monoclonal antibody of IgG2b (Chemicon International Inc., cat. No. MAB3032) that recognizes both single and double stranded DNA. It was prepared from mouse ascites by using the *calif thymus* DNA as an immunogen. The total protein concentration of this antibody solution as purchased is 25 g/L and about 10% of the protein is anti-DNA antibody.

A 68 bp double stranded DNA (ds-DNA) labeled with ^{35}S , and the anti-DNA antibody were mixed at an appropriate ratio and the resulting solution was incubated for 30 minutes at 37°C to prepare the masked anti-DNA antibody. The sequence of the 68 bp ds-DNA is given below. The amount of the anti-DNA antibody used was 33 fmol, and that of the 68 bp ds-DNA used for masking the active sites was 2 ~ 120 fmol. The MES buffer at pH 6.0 was used in this masking reaction. The 68 bp ds-DNA labeled with a ^{35}S β emitter was prepared by PCR by adding about 2% mole fraction of α - ^{35}S -dATP relative to the total dNTP.

KS primer

5' CGAGGTCGACGGTATCGATAAAAGAAAAGAAAGAATTCAAGAAAAGAAA
AGGA TCCACTAGTTCTAGA 3'

3' GCTCCAGCTGCCATAGCTATTTCTTTTCTTTCTTAAGTTCTTTTCTTTTCCT
AGGTGATCAAGATCT 5'

SK primer

b) Formation of the monolayer of thiol molecules on the surface of the Au substrate and introduction of the reaction groups

The Au substrate used was a glass plate of 12.7 mm×12.7 mm size on which Au was

vacuum-deposited to about 1000 Å thickness. In order to ensure the cleanness of the surface of the Au thin film, it was washed with Piranha solution for 10~15 minutes at 60~70°C right before using and was rinsed with deionized water and subsequently with absolute ethanol.

5 As a thiol molecule having a non-reactive group, 1-heptanethiol was used. A mixed monolayer of 11-mercaptopundodecanoic acid and 1-heptanethiol was formed as in Example 1. A 9 mm diameter portion of the Au thin film was exposed to 300 µl of a 2 mM mixed thiol solution in ethanol for 2 hours at room temperature to introduce the carboxyl reaction group, and it was then washed with absolute ethanol.

10 c) Activation of the carboxyl reaction group on the monolayer of the thiol molecules

The Au thin film where the carboxyl reaction groups were introduced was placed in 300 µl of a buffer solution (pH 6.0 MES buffer) containing 10 mM of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 5 mM of N-hydroxysulfosuccinimide (sulfo-NHS) for 2 hours at room temperature, with a 9 mm diameter portion of the Au film exposed. The carboxyl group was reacted with sulfo-NHS in the presence of EDC to form sulfo-NHS-ester (J. V. Staros, R. W. Wright, and D. M. Swingle, *Anal. Biochem.*, vol.156, pp.220-222, 1986), thereby being activated.

20 d) Immobilization reaction of anti-DNA antibody

After activating the carboxyl group on the monolayer, the reaction solution was removed and the Au substrate was placed in the solution of the masked anti-DNA antibody to carry out the immobilization reaction. The total amount of the anti-DNA antibody used was about 33 fmol. In this step, the activated carboxyl (sulfo-NHS-ester) on the supporting

material reacted with the primary amine (-NH₂) of the protein to form amide bond (-CO-NH-) (J. V. Staros, R. W. Wright, and D. M. Swingle, *Anal. Biochem.*, vol.156, pp.220-222, 1986; V. M. Mirsky, M. Riepl, and O. S. Wolfbeis, *Biosens. Bioelectron.*, vol.12, pp.977-989, 1997). As a result, the protein was immobilized on the supporting material. The immobilization reaction was carried out in the MES buffer at pH 6 for 2 hours at 10°C. The MES buffer contained the ³⁵S labeled 68 bp ds-DNA used to mask the active sites. About 33 fmol of the anti-DNA antibody and about 30 fmol of the 68 bp ds-DNA used for masking the active sites were added to 100 µl of the immobilization reaction solution.

Example 3: Measurement of the activity of the immobilized *Taq* DNA polymerase

In order to measure the activity of the immobilized *Taq* DNA polymerase, PCR was carried out and the amount of the amplified DNA was quantified. PCR was carried out in a Model 480 PCR thermal cycler of Perkin Elmer.

The 65 base ss-DNA shown in Example 1 was used as a template, and the KS primer and the SK primer were used as primers for PCR. The volume of the PCR solution used was 50 µl, and 25 fmol of the 65 base ss-DNA and 10 pmol each of the KS primer and the SK primer were added. As a buffer solution, the pH 8.3, 10X buffer purchased from Perkin Elmer was used after diluting 10 times. The temperature cycle was set as follows:

Hot start step: 94 °C, 10 minutes

PCR cycle (20-45 cycles): 94 °C, 30 s; 50 °C, 60s; 72 °C, 30s

For quantification of the DNA amplified by the PCR, 20 µl of the PCR solution was sampled and analyzed by agarose gel electrophoresis. The PCR products were visualized by fluorescence from ethidium bromide staining, and the PCR product bands were quantified with a densitometer.

Example 4: Activity of the immobilized *Taq* DNA polymerase as a function of the mole fraction of the carboxyl reaction group

The immobilization reaction was carried out in a phosphate buffer at pH 8.3 for 30 minutes at 50°C. 0.75 pmol of the *Taq* DNA polymerase and 1.5 pmol of the masking DNA were added to 50 µl of the immobilization reaction solution. 0.75 pmol of the *Taq* DNA polymerase corresponds to the amount that can form three monolayers on the area of 3 mm × 5 mm of the Au substrate. 35 cycle PCR was carried out with the immobilized *Taq* DNA polymerase and the resulting activity was measured.

Figure 1a shows agarose gel fluorescence photographs of the PCR products. The leftmost lanes show ds-DNA molecular weight marker, and the rightmost lanes show the PCR products amplified with one monolayer amount of the solution phase *Taq* DNA polymerase. Other lanes show the PCR products resulted from the immobilized *Taq* DNA polymerase. The numbers under the bottom of each lane are the mole fractions of 11-mercaptoundecanoic acid relative to the total amount of the thiol molecules used.

The activity obtained from the fluorescence photographs of Figure 1a is shown in Figure 1b. The x-axis is the mole fraction of the thiol molecule having the carboxyl reaction group, relative to the total moles of the thiol molecules used. The y-axis is the relative activity of the immobilized *Taq* DNA polymerase, as compared to the activity of one monolayer amount of the solution phase *Taq* DNA polymerase. The solid circle denotes the results of immobilization when the active site was masked (PIM) and the open circle denotes those of immobilization when the active site was not masked (RIM).

In the whole range of the mole fraction, the PIM in which the active site was masked shows higher activity than the RIM in which the active site was not masked. Also, it can be

seen that the activity of the masked DNA polymerase is the highest when the mole fraction is about 5%. This demonstrates that the activity preservation of the masked DNA polymerase can be maximized kinetically by controlling the mole fraction of the carboxyl reaction group on the substrate material. This result shows that the activity of the immobilized enzyme can be maximized by masking the active site and also by kinetically preventing formation of multiple immobilization bonding that causes reduction or damage of the activity.

Example 5: Activity of the immobilized *Taq* DNA polymerase as a function of the masking ratio of the active site

The number of moles of the partially double stranded DNA used to mask the active site relative to that of the *Taq* DNA polymerase used was varied from 0 to 2, and the activity of the immobilized *Taq* DNA polymerase was measured. The results are shown in Figures 2a and 2b. In Figure 2a, the leftmost and rightmost lanes are the same as in Figure 1a, and other lanes are the results of the PCR products amplified with the immobilized *Taq* DNA polymerase at different masking ratio. The numbers given below are the % ratio corresponding to the number of moles of the partially double stranded DNA used for masking relative to that of the *Taq* DNA polymerase.

The activity of the immobilized enzyme is shown as a relative activity with respect to the activity in the solution phase as in Figure 1b. The molar amount of 11-mercaptoundecanoic acid with respect to the total moles of the thiol molecules used for introducing the carboxyl reaction group on the Au surface was 5.0%. The total amount of the *Taq* DNA polymerase used for the immobilization reaction was 0.75 pmol that corresponded to three monolayers as in Figure 1b. Other reaction conditions for

immobilization and PCR were the same as in Example 4. Figures 2a and 2b demonstrate that the active site masking occurs by forming a 1:1 complex of the partially double stranded DNA and the *Taq* DNA polymerase.

5 Example 6: Activity of the immobilized *Taq* DNA polymerase as a function of the immobilization pH

10 The activity of the immobilized DNA polymerase was measured at different immobilization pH, while keeping at 5.0% the mole fraction of 12-mercapdodecanoic acid with respect to the total moles of the thiol molecules used for introducing the carboxyl reaction group on the Au surface. Other reaction conditions for immobilization and PCR were the same as in Example 4. The results are shown in Figures 3a and 3b. The leftmost and rightmost lanes in Figure 3a are the same as in Figure 1a, and other lanes are the results of the PCR products amplified with the immobilized *Taq* DNA polymerase at different immobilization pH. The pH of the buffer solution used in the immobilization reaction are shown on the bottom of each lane. Figure 3a and 3b show that the masking efficiency of the active site is maximized at pH 8.3 where the binding efficiency of the *Taq* DNA polymerase is known to be maximum.

15

20 Example 7: Activity of the immobilized *Taq* DNA polymerase as a function of the immobilization reaction time

 The activity of the immobilized DNA polymerase was measured at different immobilization reaction time, while keeping at 5.0% the mole fraction of 12-mercapdodecanoic acid with respect to the total moles of the thiol molecules used for introducing the carboxyl reaction group on the Au surface. Other reaction conditions for

immobilization and PCR were the same as in Example 4. The results are shown in Figures 4a and 4b. The leftmost and rightmost lanes in Figure 3a are the same as in Figure 1a. Other lanes are the results of the PCR products amplified with the immobilized *Taq* DNA polymerase at different immobilization reaction time. The immobilization reaction time is indicated in the unit of minute at the bottom of each lane.

The rapid increase observed at the reaction time shorter than about 10 minutes in Figure 4a and 4b suggests that the probability of immobilizing *Taq* DNA polymerase increases as the reaction time while the probability of forming multiple immobilization bonding is kept low. The slow decrease in the region of the reaction time longer than 10 minutes results from reduction in the activity due to the formation of multiple immobilization bonding as well as the spatial restriction caused by increased number density of the immobilized enzyme. These results suggest that the overall activity per unit area of immobilization can be maximized by optimizing the immobilization reaction time, which is of particular importance kinetically, thereby achieving both high probability of immobilization and suppression of the probability of forming multiple immobilization bonding.

Example 8: Comparison of solution phase and immobilized *Taq* DNA polymerase as a function of number of cycles of PCR

The activity of the immobilized DNA polymerase was measured at different number of cycles of PCR, while keeping at 5.0% the mole fraction of 12-mercapdodecanoic acid with respect to the total moles of the thiol molecules used for introducing the carboxyl reaction group on the Au surface. Other reaction conditions for immobilization and PCR were the same as in Example 4. The results are shown in Figure 5a and 5b. In Figure 5a, the

number of cycles of PCR is given at the bottom of each lane.

Figures 5a and 5b show that the trend observed in the activity of the immobilized *Taq* DNA polymerase is nearly identical to that of the solution phase *Taq* DNA polymerase. This suggests that the activity preservation ratio per immobilized molecule is maximized, i.e., the activity of the immobilized enzyme being close to the solution phase.

Example 9: Activity of the immobilized *Taq* DNA polymerase as a function of total amount of *Taq* DNA polymerase used

The activity of the immobilized DNA polymerase was measured at different amount of *Taq* DNA polymerase corresponding to 0 to 10 monolayers, while keeping at 5.0% the mole fraction of 12-mercapdodecanoic acid with respect to the total moles of the thiol molecules used for introducing the carboxyl reaction group on the Au surface. The number of moles of the partially double stranded DNA used for masking the active site was twice that of the *Taq* DNA polymerase. Other reaction conditions for immobilization and PCR are the same as in Example 4. The results are shown in Figures 6a and 6b. The leftmost and rightmost lanes are the same as in Figure 1a, and other lanes are the results of the PCR products for different amount of *Taq* DNA polymerase used. The amount of *Taq* DNA polymerase is shown in the unit of monolayer at the bottom of each lane.

Figures 6a and 6b show that the activity of the immobilized enzyme can be increased by controlling the amount of the *Taq* DNA polymerase used.

Example 10: Measurement of activity of the immobilized anti-DNA antibody

The activity of the immobilized anti-DNA antibody was measured using a β -counter (Beckman, Model LS6500) by counting β -emission from the ^{35}S labeled 68 bp ds-DNA

used for masking the active sites. The β -emission measurements were performed with the antibody immobilized Au film placed in 2 ml of the scintillation cocktail.

Example 11: Activity of the immobilized anti-DNA antibody as a function of the mole
5 fraction of the carboxyl reaction group on the supporting material

As in the case of the *Taq* DNA polymerase, in the whole range of the mole fraction, the PIM in which the active sites were masked shows higher activity than the RIM in which the active sites were not masked. Also it can be seen that the activity of the PIM is the highest when the mole fraction is about 8%. This demonstrates that the activity preservation of the
10 masked antibody can be maximized kinetically by controlling the mole fraction of the carboxyl reaction group on the substrate material. This results show that the activity of immobilized antibody can be maximized by masking the active site and also by kinetically preventing formation of multiple immobilization bonding that causes reduction or damage of the activity.

15 The x-axis in Figure 7 is the same as that in Figure 1b, and the y-axis is the activity of the immobilized antibody that is measured by detecting β -emission from the ^{35}S labeled ds-DNA bound to the antibody. The solid circle denotes the results of immobilization when the active sites were masked (PIM) and the open circle denotes those of immobilization when the active sites were not masked (RIM).

20 Example 12: Activity of the immobilized anti-DNA antibody as a function of the concentration of the antigenic ds-DNA.

The change in the activity of the immobilized anti-DNA antibody as a function of the concentration of the ^{35}S labeled 68 bp ds-DNA is shown in Figure 8. The activity of the

immobilized anti-DNA antibody was measured at different concentrations of the 68 bp ds-DNA used for masking. The total amount of the anti-DNA antibody used for immobilization reaction was about 33 fmol. The mole fraction of the 11-mercaptoundodecanoic acid used to introduce carboxyl reaction group on the Au surface with respect to the total moles of the thiol molecules was 10%. Other reaction conditions for immobilization are the same as in Example 11, except for the number of moles of the 68 bp ds-DNA.

In Figure 8, the solid and open circles denote the PIM and the RIM, respectively. The PIM case shows higher activity than the RIM. The saturation phenomenon was observed in the PIM case when the molar ratio of the anti-DNA antibody to the 68 bp ds-DNA used for masking was in the range 1:1 ~ 1:2. This demonstrates that the active sites were masked by formation of the antigen-antibody complex.

WHAT IS CLAIMED IS:

1. A method for immobilizing a physiologically active molecule on a substrate material comprising the steps of :

5 (a) reacting the physiologically active molecule with a masking compound that selectively binds to the active so as to mask the active site;

(b) forming a supporting material by introducing on the substrate material a linker that will bind to the masked physiologically active molecule prepared in step (a);

10 (c) controlling the rate of the immobilization reaction in which the masked physiologically active molecule prepared in step (a) binds to the linker on the supporting material formed in step (b); and

(d) immobilizing the masked physiologically active molecule prepared in step (a) on the supporting material by reacting with the linker on the supporting material formed in step (b).

15 2. The method of Claim 1, wherein step (b) comprises a step of forming a thin film of the linker and a step of controlling the molar ratio of the reaction group of the linker on the supporting material by controlling the ratio of the linker having the reaction group to a non-reactive linker having a non-reactive group.

20 3. The method of Claim 1, wherein step (c) comprises a step of controlling concentration of the masked physiologically active molecule.

4. The method of Claim 1, wherein step (c) comprises a step of controlling pH.

5. The method of Claim 1, wherein step (c) comprises a step of controlling reaction time.

5 6. The method of Claim 1, wherein step (c) comprises a step of controlling reaction temperature.

7. The method of Claim 1, which further comprises a step of activating the reaction group of the linker by using a coupling reagent.

10

8. The method of Claim 1, wherein the physiologically active molecule is protein, enzyme, antigen, or antibody.

15

9. The method of Claim 1, wherein the making compound that selectively binds to the active site is one selected from the group consisting of substrate, inhibitor, cofactor, or their chemically modified compound, their homolog, and their derivative for masking enzyme; or it is one selected from the group consisting of corresponding antibody, antigen, and their modifications for masking antigen or antibody.

20

10. The method of Claim 1, wherein the active site is one or more active sites or one or more cofactor sites of the physiologically active molecule.

11. The method of Claim 1, wherein the masking compound that selectively binds to the physiologically active molecule binds through covalent bonding, ionic bonding,

coordination bonding, hydrogen bonding, dipole-dipole interaction, packing, or their combination.

12. The method of Claim 1, wherein the masking ratio of the physiologically active molecule is 5 ~ 100%.

13. The method of Claim 1, wherein the substrate material is metal, non-metal, semiconductor, oxide of these elements, organic or inorganic macromolecule, dendrimer, or their mixture; and it is of a planar type, a spherical type, a linear type, a porous type, a microfabricated gel pad, or a nano-particle.

14. The method of Claim 1, wherein the linker in step (b) forms a thin film of the linker on the substrate material through covalent bonding, ionic bonding, coordination bonding, hydrogen bonding, packing, or their combination.

15. The method of Claim 14, wherein the reaction group of the linker that reacts with the substrate material are thiol, sulfide, disulfide, silane, carboxyl, amine, alcohol, aldehyde, epoxy, alkyl halide, alkyl, alkene, alkyne, aryl, or their combination.

16. The method of Claim 1, wherein the reaction group of the linker that reacts with the physiologically active molecule is carboxyl, amine, alcohol, aldehyde, epoxy, thiol, sulfide, disulfide, alkyl halide, alkyl, alkene, alkyne, aryl, or their combination.

17. The method of Claim 1, wherein the physiologically active molecule and the

reaction group of the linker are connected by covalent bonding, ionic bonding, coordination bonding, hydrogen bonding, packing, or their combination.

18. The method of Claim 1, wherein the physiologically active molecule and the
5 reaction group of the linker are connected by amide bonding, imine bonding, sulfide bonding, disulfide bonding, ester bonding, ether bonding, amine bonding, or their combination.

19. The method of Claim 18, wherein the physiologically active molecule and the
10 reaction group of the linker are connected by amide bonding.

20. The method of Claim 18, wherein a carboxyl group of the physiologically active molecule and an amine reaction group of the linker are connected by amide bonding.

15 21. The method of Claim 18, wherein an amine group of the physiologically active molecule and an aldehyde reaction group of the linker are connected by imine bonding.

22. The method of Claim 18, wherein an aldehyde group of the physiologically active molecule and an amine reaction group of the linker are connected by imine bonding.

20 23. The method of Claim 18, wherein a thiol group of the physiologically active molecule and a thiol reaction group of the linker are connected by disulfide bonding.

24. The method of Claim 2, wherein the linker having the reaction group is one selected

from the group consisting of mercaptocarboxylic acid, mercaptoaminoalkane, mercaptoaldehyde, dimercaptoalkane, and sulfide and disulfide having a reaction group such as carboxyl, thiol, alcohol, aldehyde, and amine; and the non-reactive linker having the non-reactive group is one selected from the group consisting of mercaptoalkane, mercaptoalcohol, sulfide, and disulfide.

25. The method of Claim 24, wherein the linker having the reaction group is mercaptocarboxylic acid or mercaptoaminoalkane, and the non-reactive linker having the non-reactive group is mercaptoalcohol or mercaptoalkane.

26. The method of Claim 24, wherein the linker having the reaction group is mercaptoaldehyde, and the non-reactive linker having the non-reactive group is mercaptoalcohol or mercaptoalkane.

27. The method of Claim 24, wherein the linker having the reaction group is dimercaptoalkane, and the non-reactive linker having the non-reactive group is mercaptoalcohol or mercaptoalkane.

28. The method of Claim 24, wherein the mercaptocarboxylic acid is 11-mercaptoundodecanoic acid.

29. The method of Claim 24, wherein the mercaptoalcohol is 6-mercapto-1-hexanol and the mercaptoalkane is 1-heptanethiol.

30. The method of Claim 3, wherein the linker having the reaction group is 0.05~50% of the total linker.

31. The method of Claim 30, wherein the linker having the reaction group is 0.05 ~
5 30% of the total linker.

32. The method of Claim 1, which further comprises step (e) of removing the masking compound from the masked physiologically active molecule immobilized in step (d).

10 33. A masked physiologically active molecule immobilized on a supporting material made according to the method of any of Claims 1 through 32.

34. A physiologically active molecule immobilized on a supporting material made according to the method of Claim 32.

1/8

Fig. 1a

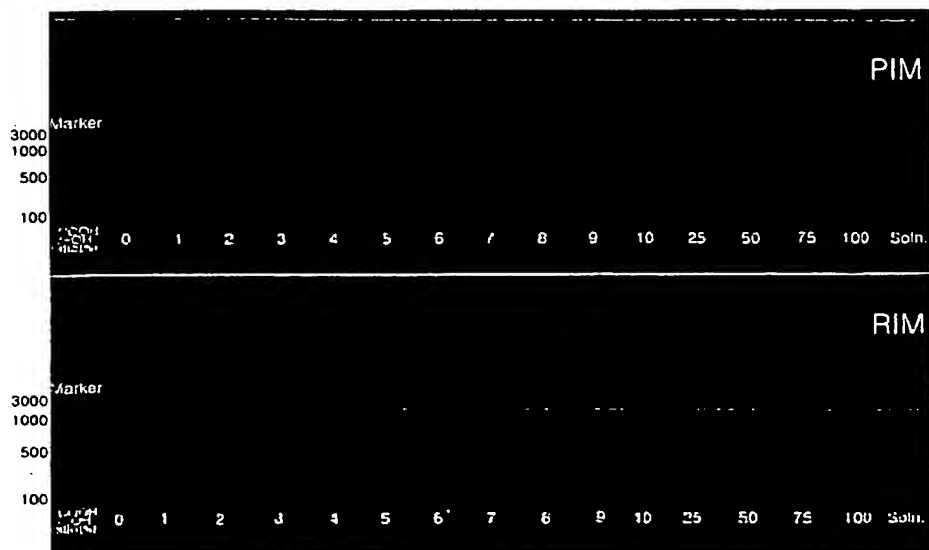
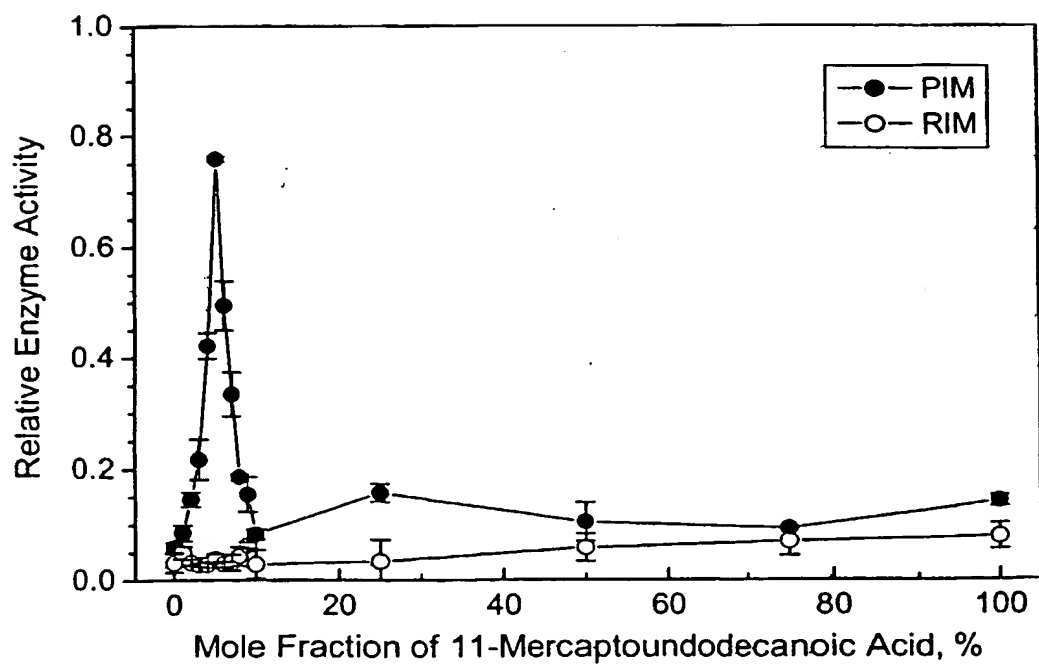
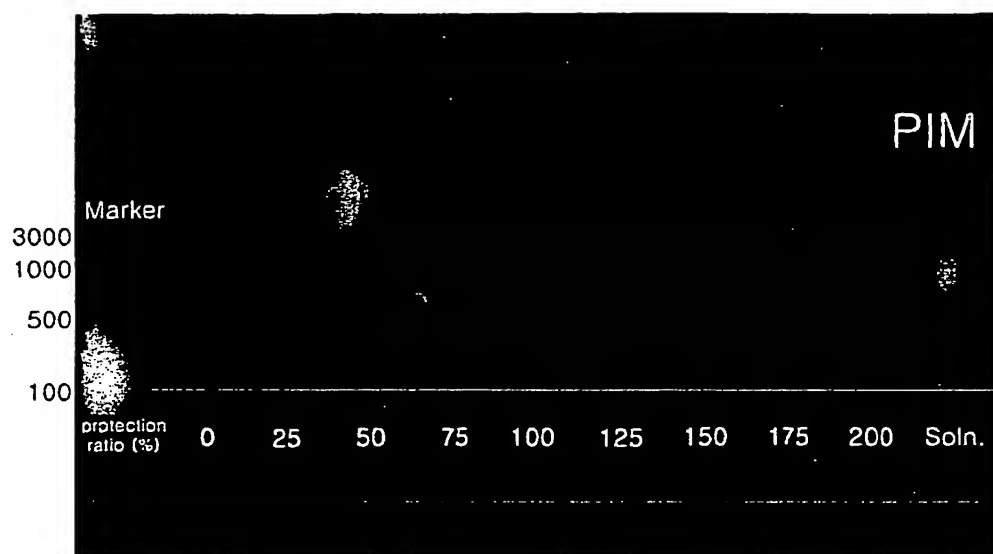
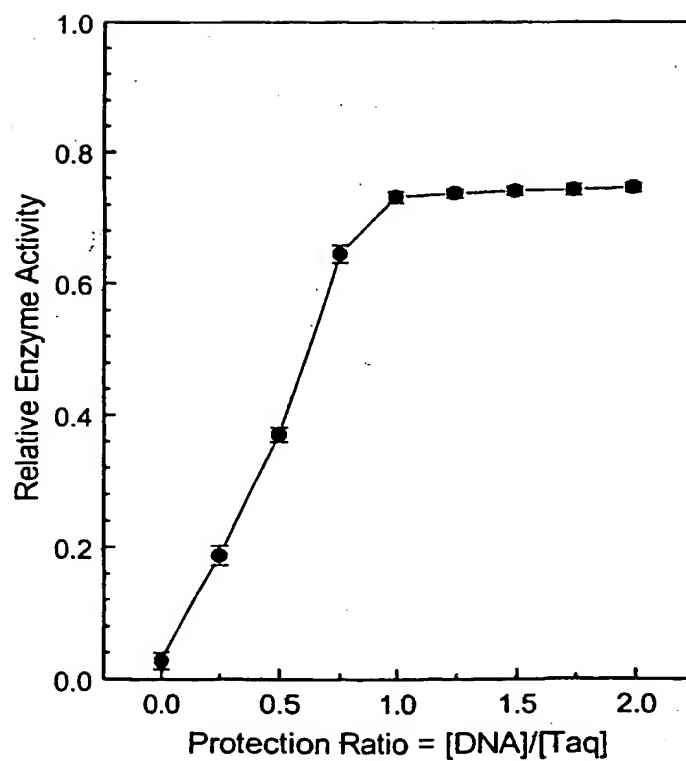


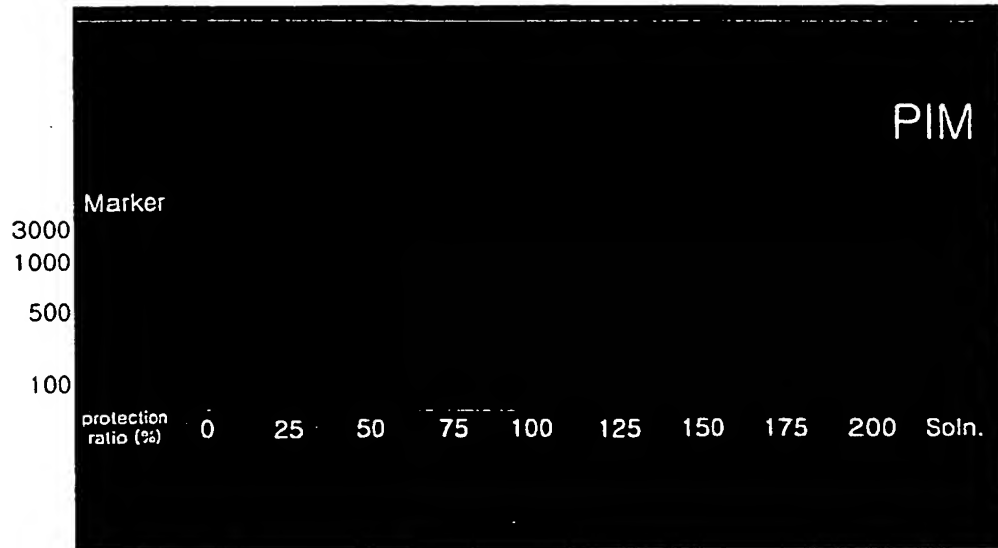
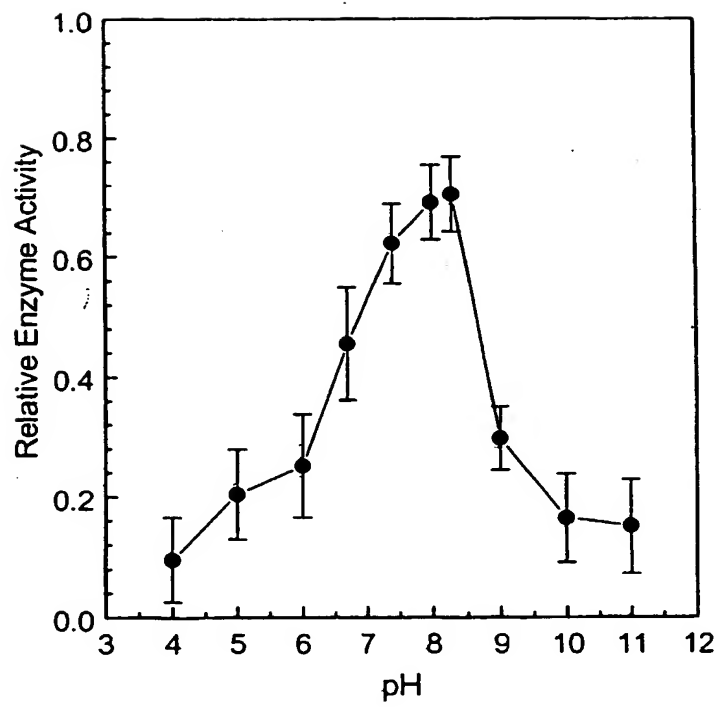
Fig. 1b



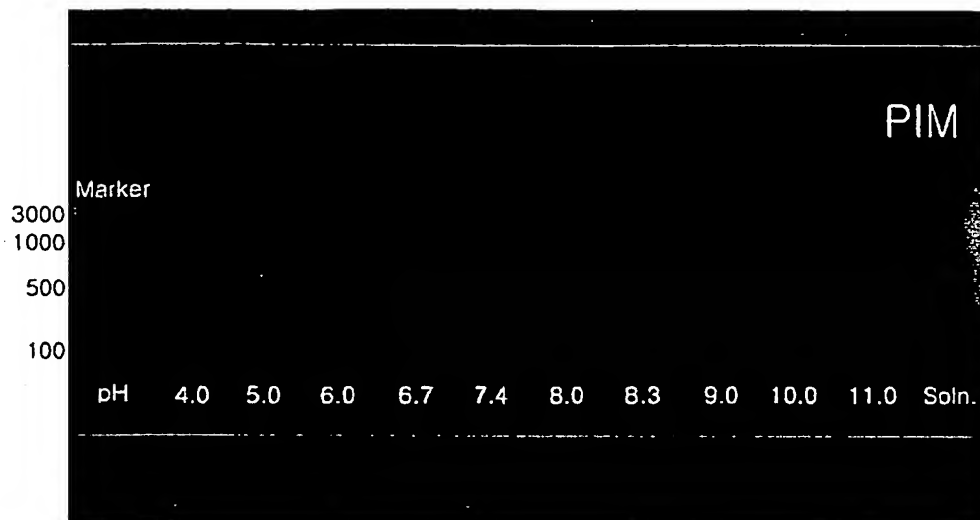
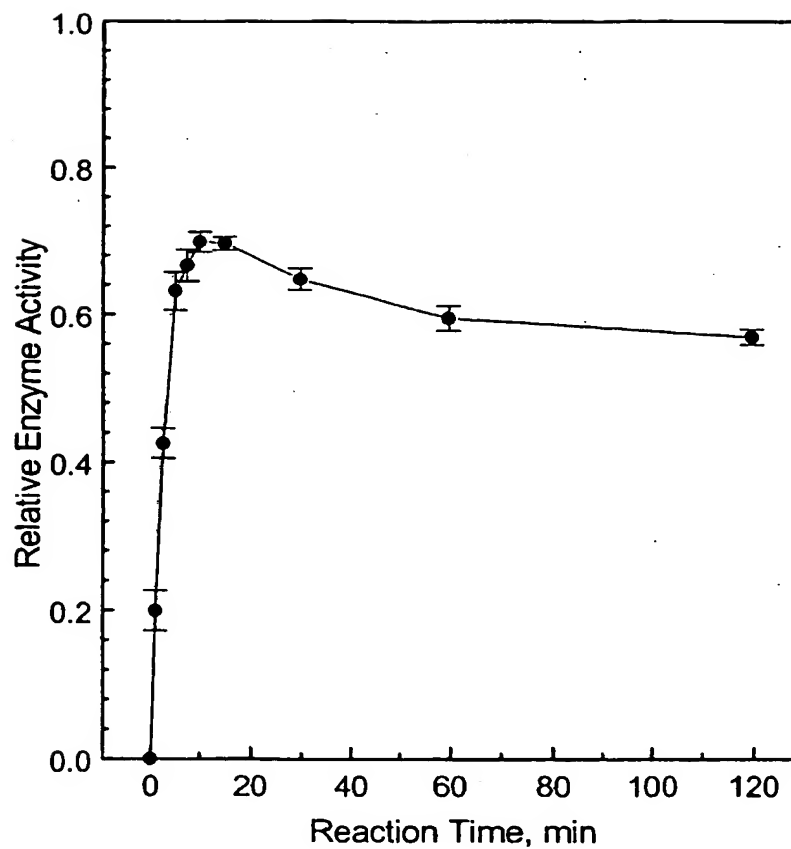
2/8

Fig. 2a**Fig. 2b**

3/8

Fig. 3a**Fig. 3b**

4/8

Fig. 4a**Fig. 4b**

5/8

Fig. 5a

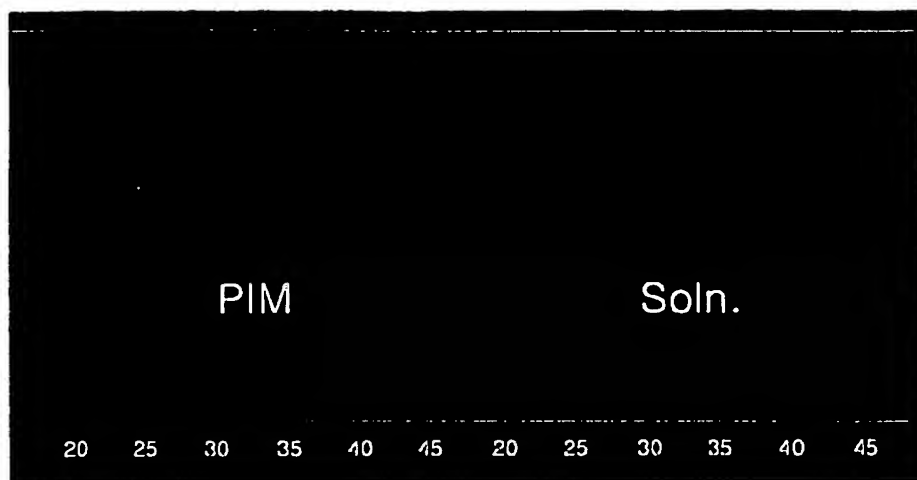
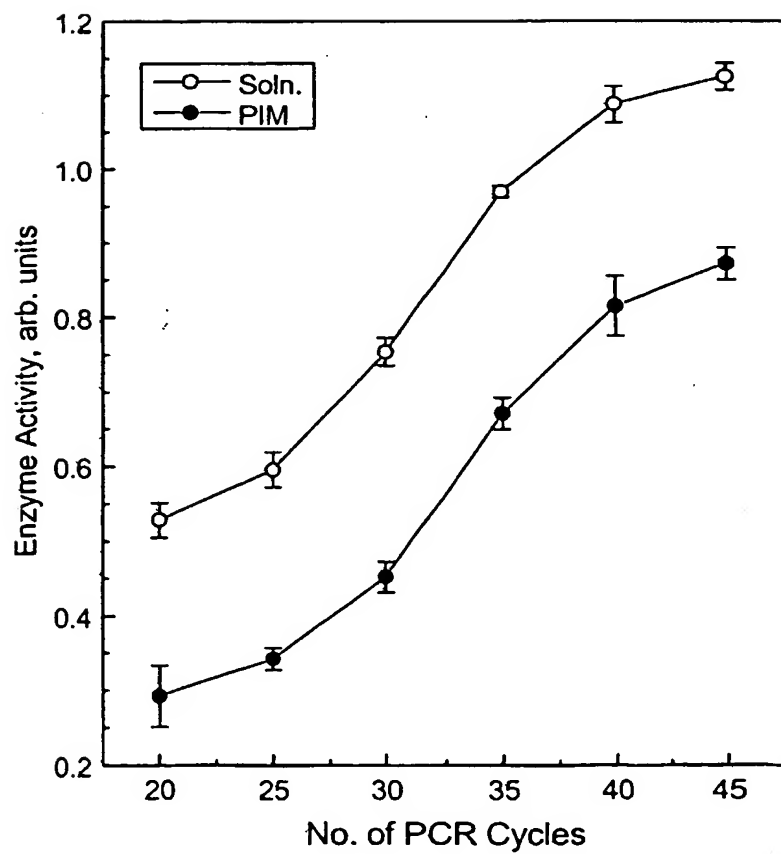
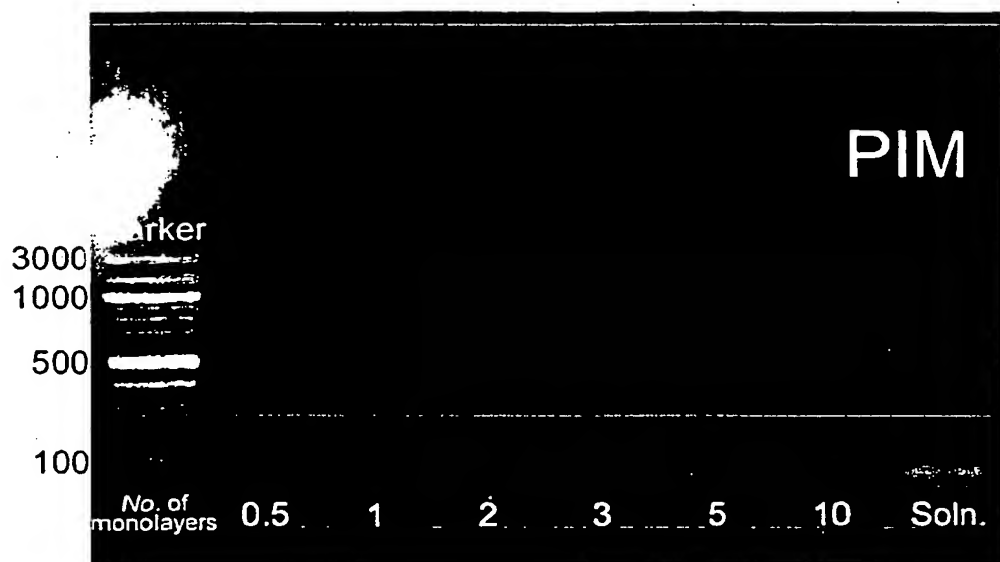
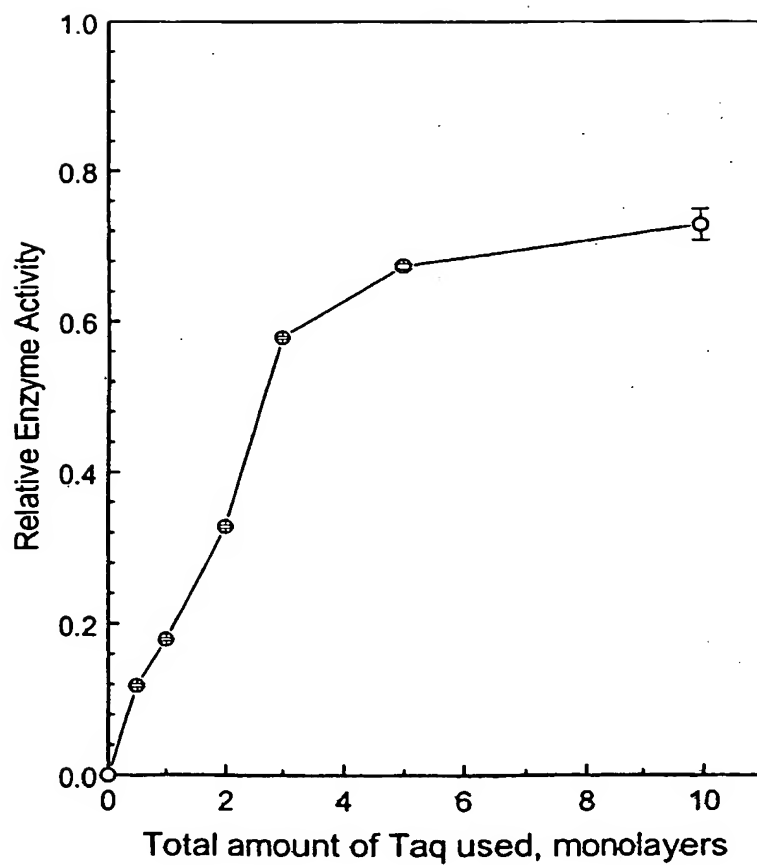


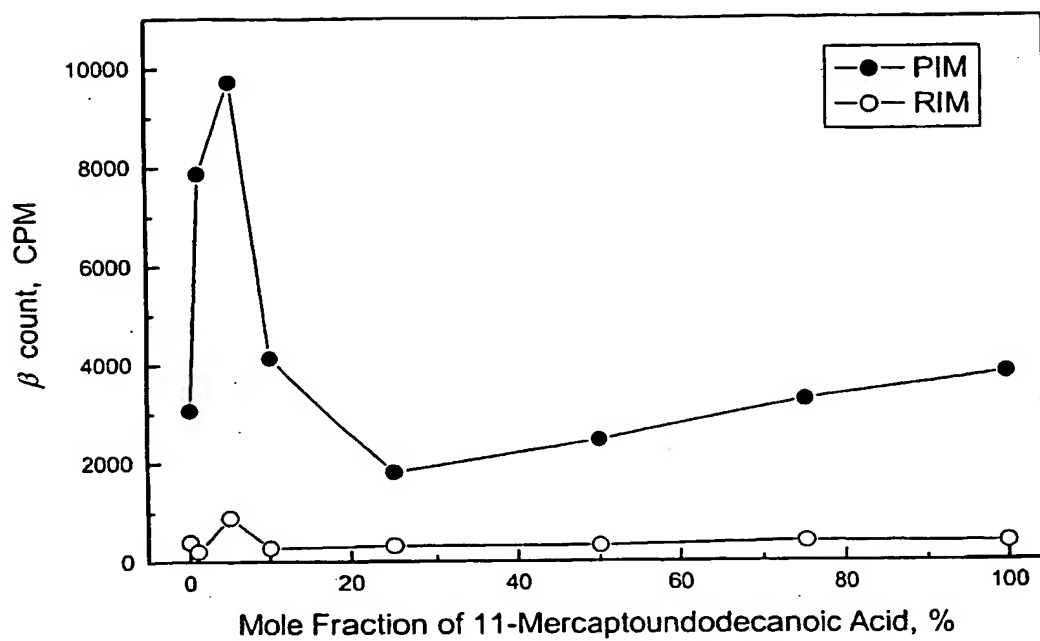
Fig. 5b



6/8

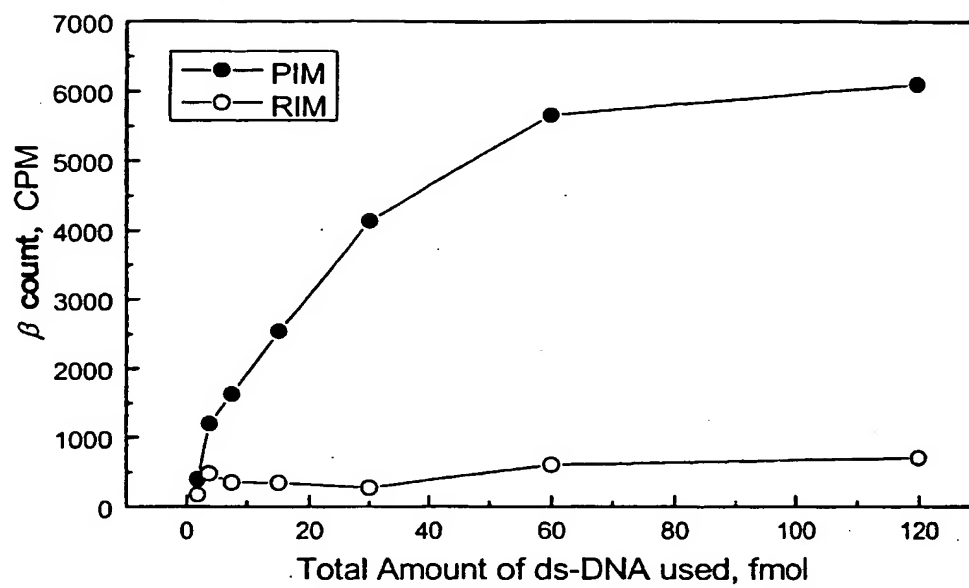
Fig. 6a**Fig. 6b**

7/8

Fig. 7

8/8

Fig. 8



INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR01/01239

A. CLASSIFICATION OF SUBJECT MATTER**IPC7 C12N 11/00**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N 11/00, C12N 11/02, C12N 11/14

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Espasenet, PAJ, USPTO, CA, Pubmed; "masking", "immobilization", "linker"

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	Kumakura, M. et al., "Immobilization of enzymes masks their active site", Biosci. Rep., 4, 181-87, 1984. see abstract	1-34
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Y	Webster, I.A., et al., "Using the rotational masking concept to enhance substrate-inhibited reaction rates : controlled pore supports for enzyme immobilization", Enzyme Microb. Technol., 7(6), 266-74, 1985.	1-34

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

11 APRIL 2002 (11.04.2002)

Date of mailing of the international search report

12 APRIL 2002 (12.04.2002)

Name and mailing address of the ISA/KR

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Authorized officer

LEE, Cheo Young

Telephone No. 82-42-481-5594



INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR01/01239

Patent document
cited in search reportPublication
datePatent family
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